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Chitra Krishnamurti, Svetislava J. Vukelja and Barbara M. Alving				
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Accession For NTIS CRA&I <input checked="" type="checkbox"/> Unannounced <input type="checkbox"/> Justification By Distribution/ Availability Codes Dist Avail and/or Special A-1 20 DTIC QUALITY INSPECTED 3		INHIBITORY EFFECTS OF LYSINE ANALOGUES ON t-PA INDUCED WHOLE BLOOD CLOT LYSIS Abstract: The lysine analogues epsilon-aminocaproic acid (EACA) and trans-4-amino-methyl cyclohexane carboxylic acid (AMCA) are used to prevent excessive bleeding in patients with coagulopathies, such as hemophilia and thrombocytopenia, or in those who have received tissue plasminogen activator (t-PA). However, their relative efficacy in inhibiting lysis of clots that have been formed in the presence of exogenous t-PA or that have been formed and then exposed to exogenous t-PA has not been well characterized. The present study utilized blood from normal volunteers and ¹²⁵ I-fibrinogen in a dilute whole blood clot assay to determine the relative concentrations of lysine analogues required for inhibition of clot lysis induced by exogenous t-PA. AMCA (0.05 mM) and EACA (0.6 mM) were effective in prolonging clot lysis if (1) whole blood clots were formed and then exposed to a lysine analogue and exogenous t-PA or if (2) whole blood clots were formed in the presence of exogenous t-PA and a lysine analogue. However, their inhibitory effect was markedly reduced if clots were formed in the presence of t-PA and then exposed to either of the lysine analogues. The analogues did not inhibit the initial binding of t-PA to fibrin. They did inhibit binding of plasminogen to fibrin as well as the activation of plasminogen by t-PA in the absence of fibrin. The data suggest that lysine analogues, even at low concentrations, reduce the rate of t-PA induced whole blood clot lysis by several mechanisms.		
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INHIBITORY EFFECTS OF LYSINE ANALOGUES ON t-PA INDUCED WHOLE BLOOD CLOT LYSIS

Chitra Krishnamurti, Svetislava J. Vukelja¹ and Barbara M. Alving

Walter Reed Army Institute of Research, Washington, DC and
¹Brooke Army Medical Center, Fort Sam Houston, TX

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Abstract: The lysine analogues epsilon-aminocaproic acid (EACA) and trans-4-amino-methyl cyclohexane carboxylic acid (AMCA) are used to prevent excessive bleeding in patients with coagulopathies, such as hemophilia and thrombocytopenia, or in those who have received tissue plasminogen activator (t-PA). However, their relative efficacy in inhibiting lysis of clots that have been formed in the presence of exogenous t-PA or that have been formed and then exposed to exogenous t-PA has not been well characterized. The present study utilized blood from normal volunteers and ¹²⁵I-fibrinogen in a dilute whole blood clot assay to determine the relative concentrations of lysine analogues required for inhibition of clot lysis induced by exogenous t-PA. AMCA (0.06 mM) and EACA (0.6 mM) were effective in prolonging clot lysis if (1) whole blood clots were formed and then exposed to a lysine analogue and exogenous t-PA or if (2) whole blood clots were formed in the presence of exogenous t-PA and a lysine analogue. However, their inhibitory effect was markedly reduced if clots were formed in the presence of t-PA and then exposed to either of the lysine analogues. The analogues did not inhibit the initial binding of t-PA to fibrin. They did inhibit binding of plasminogen to fibrin as well as the activation of plasminogen by t-PA in the absence of fibrin. The data suggest that lysine analogues, even at low concentrations, reduce the rate of t-PA induced whole blood clot lysis by several mechanisms.

Key Words: lysine analogues, whole blood clot lysis, tissue plasminogen activator, fibrin.

Corresponding Author: Dr. Chitra Krishnamurti, Department of Hematology, Walter Reed Army Institute of Research, Washington, DC - 20307-5100.
Tel. No. - (202) 576-3385; FAX No. - (202) 576-0703

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Intravascular fibrinolysis is initiated by the conversion of plasminogen to plasmin by t-PA on a fibrin surface (1). Although t-PA can activate plasminogen to plasmin in the absence of fibrin, the catalytic efficiency is enhanced at least 100-fold when t-PA and plasminogen form a complex on fibrin (2). Human t-PA binds to fibrin initially through its finger domain; as fibrin degradation progresses, t-PA binding may also occur through the kringle 2 domain (3). Binding of plasminogen to fibrin is mediated by lysine-binding sites located in its five kringle structures (1,4). The first kringle domain has a high affinity binding site ($K_d=9 \mu\text{M}$) for the lysine analogue ϵ -aminocaproic acid (EACA) and the others have lower affinity binding sites ($K_d=5 \text{ mM}$) (5).

The lysine analogues EACA and trans-4-aminomethyl cyclohexane carboxylic acid (AMCA) have been used extensively *in vivo* to inhibit fibrinolysis in order to reduce blood loss that occurs after mucosal injury (6-11). They are also used in patients who have unacceptable bleeding while receiving fibrinolytic agents such as t-PA (12). Experimental support for this treatment has been demonstrated in an animal model (13). In this model, lysine analogues inhibited bleeding induced by lacerations of the marginal ear vein in rabbits receiving t-PA.

In order to characterize the inhibitory effects of these analogues, we have utilized the dilute whole blood clot assay as an *ex vivo* representation of the fibrinolytic system (14,15). In this assay, dilute whole blood is clotted with thrombin under conditions that result in clot retraction and in fibrin crosslinking, therefore including α_2 -antiplasmin in the clot (16). The inhibitory effect of the analogues was measured in dilute whole blood clots that had been formed in the presence of exogenous t-PA and then exposed to EACA and AMCA and in clots that were first formed and then exposed to the lysine analogues and exogenous t-PA.

MATERIALS AND METHODS

Iodogen was purchased from the Pierce, Rockford, IL; EACA was obtained from Elkins-Sinn, Inc, Cherry Hill, NJ and AMCA was purchased from Kabi Vitrum, Alameda, CA. Lysine-Sepharose and Sephadex G-25 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden and the chromogenic substrate S-2251 was purchased from KabiVitrum, Stockholm, Sweden. Single chain human recombinant tissue plasminogen activator (t-PA, specific activity 580,000 IU/mg, Activase) was purchased from Genentech, Inc., South San Francisco, CA. Human thrombin (specific activity, 4971 U/mg) was from Sigma, St. Louis, MO, and human plasmin (10.6 CTA U/mg) was a gift from the Center for Biologics Evaluation and Research, Bethesda, MD. Human plasminogen was prepared from Cohn fraction III paste (provided by the Cutter Co, Berkeley, CA) by affinity chromatography on lysine-Sepharose (17). The plasminogen was in the glu-form as judged by SDS-gel electrophoresis and had a specific activity of 20 CTA U/mg. Human fibrinogen (90% clottable) was purchased from KabiVitrum and chromatographed on lysine-Sepharose to remove plasminogen (18). Fibrinogen which was to undergo iodination was purified from plasma by the Kazal technique and was 98% clottable (19).

Human volunteers

Blood for these studies was obtained from a total of three healthy volunteers who gave

informed consent under a protocol approved by the Human Use Committee of the Walter Reed Army Institute of Research.

¹²⁵Iodination of fibrinogen

Human fibrinogen was labeled with ¹²⁵I by the iodogen procedure (20). A 2 ml solution of fibrinogen (1 mg/ml) in 0.05 M Tris-HCl, 0.10 M NaCl, 0.025 M sodium citrate, pH 7.4 was mixed with 200 μ Ci ¹²⁵I at 22 °C for 5 min in a scintillation vial precoated with 20 μ g iodogen. Unbound ¹²⁵I was separated from labeled fibrinogen by chromatography on a Sephadex G-25 column. Radioactivity was measured with a gamma counter (GammaTrac[®] 1193, TM Analytic, Inc., Elk Grove Village, IL). The efficiency of labeling was 32%; clottability of the radiolabeled fibrinogen was 95% and specific activity was 1.7×10^7 cpm/mg protein.

Plasminogen and t-PA were iodinated in a similar fashion by incubating 300 μ Ci ¹²⁵I with 0.2 mg protein in buffer containing 0.05 M Tris, 0.15 M NaCl, 0.01% Tween-80, pH 7.4 at 4 °C for 2 min in a 12x75 mm glass tube precoated with 10 μ g iodogen. The specific activity of t-PA was 3.6×10^8 cpm/mg and plasminogen 1.4×10^8 cpm/mg.

Dilute Whole Blood Clot Lysis

Freshly drawn blood (2 ml) was diluted 1:10 in cold (4 °C) phosphate buffer (53 mM Na₂HPO₄ and 12 mM KH₂PO₄, pH 7.4) that contained 0.02 μ M ¹²⁵I-fibrinogen (14,21). To 2 ml aliquots of this solution were added 20 μ l t-PA (final concentrations of 3 to 1200 IU/ml), 100 μ l EACA (0.075-0.6 mM, final), AMCA (0.0075-0.06 mM, final) or buffer, and 40 μ l thrombin (1 U/ml, final). After the addition of thrombin, the solutions were incubated for 20 min at 4 °C and then transferred to a 37 °C water bath. The 0 time point for measurement of clot lysis began with the 37 °C incubation. At this time and at fixed intervals, the radioactivity was measured in 30 μ l aliquots of the supernatants. Percent lysis was calculated as:

$$\frac{\text{cpm time } x - \text{cpm time } 0}{\text{total cpm} - \text{cpm time } 0} \times 100$$

Total cpm was the radioactivity of the sample before clotting was initiated. The cpm determined at time 0 (after clot formation) were 20% of the total cpm, indicating an 80% incorporation of ¹²⁵I fibrinogen into the fibrin clot.

In this study the term "clot lysis time" refers to the time required for the release of 50% of the total counts from the radiolabeled clot.

Functional assay of t-PA activity in the absence of fibrin

t-PA (50 μ l, 2 IU/ml final) was preincubated with 50 μ l buffer (0.05 M Tris, 0.1 M NaCl, 0.1% Triton X-100, pH 8.8) or with 50 μ l EACA (0.06, 0.6 mM) or AMCA (0.006, 0.06 mM) for 10 min at 37 °C. The mixture was added to 50 μ l plasminogen (0.1 mg/ml, final) and plasmin activity was monitored by the addition of 50 μ l S-2251 (0.25 mM, final) in a 96-well plate. The change in absorbance at A405 was measured by a Titertek Multiscan spectrophotometer (Flow Laboratories, McLean, VA).

The ability of EACA or AMCA to directly inhibit the amidolytic activity of plasmin was

studied by preincubating 50 μ l plasmin (0.01 U/ml, final) with Tris-saline-triton buffer, pH 8.8 (100 μ l), or lysine analogue for 10 min at 37 °C, followed by the addition of S-2251 (50 μ l, 0.25 mM, final). At two hours the change in absorbance was recorded for plasmin alone, and for EACA (0.06, 0.6 mM) as well as for AMCA (0.006, 0.06 mM). To assess the inhibitory effect of the analogues toward the proteolytic activity of plasmin, fibrinogen (600 μ l, 0.2 mg/ml in phosphate buffer) was mixed with 125 I-fibrinogen (80 μ l) and thrombin (20 μ l, 5 U), in a 12x75 mm glass tube and the clot was wound on a glass rod. To the supernatant was added plasmin (100 μ l, 0.25 CTA U) and either 200 μ l buffer, or EACA (0.6 mM, final) or AMCA (0.06 mM, final). Aliquots (10 μ l) were then sampled during 50 min and the results expressed as per cent clot lysis. The assays were performed in triplicate at 22 °C.

Binding of radiolabeled t-PA and plasminogen to fibrin

Plasminogen-free fibrinogen (1 mg/ml, 0.2 ml) was added to each well of a 24-well plate (Falcon 3047, Becton Dickinson and Company, Lincoln Park, NJ) (22). Thrombin (10 μ l, 40 U/ml) was added and the plates were dried at 22 °C for 1 hr and subsequently at 37 °C overnight. A solution (200 μ l) of 125 I-t-PA (0.3-17 nM) or 125 I-plasminogen (0.6-15 nM) in phosphate buffered saline with 0.01% Tween-80, pH 7.4 was incubated with the fibrin-coated wells for 1 hr at 37 °C. The solution contained either no lysine analogues or EACA (0.06, 0.6 mM) or AMCA (0.06, 0.006 mM). The radioactivity of the unbound protein was measured in the supernatant. The matrix was then washed 5 times with the incubation buffer containing 10 mg/ml of human serum albumin, dissolved in 0.2 ml alkaline urea, and the radioactivity of the bound protein was measured.

Statistics

All values are expressed as the mean \pm SEM. Student's *t*-test was performed to assess statistical significance.

RESULTS

Effect of Lysine Analogues on t-PA and plasminogen

Before performing studies with dilute whole blood clots, we measured the effects of lysine analogues on the activation of plasminogen by t-PA in the absence of fibrin. EACA and AMCA at concentrations of 0.06 mM and of 0.006 mM, respectively, caused significant inhibition of activation of plasminogen by t-PA as determined by measuring the rate of conversion of plasminogen to plasmin with the substrate S-2251 (Fig. 1). Since neither of these analogues at the concentrations shown in Fig. 1 inhibited the amidolytic activity of plasmin toward S-2251, the inhibitory effect was due to decreased activation of plasminogen by t-PA. The amidolytic activity of plasmin alone did not differ by more than 3% from the activity of plasmin in the presence of EACA (0.06 mM and 0.6 mM) or AMCA (0.006 mM and 0.06 mM).

The inhibitory effect of the analogues toward the proteolytic activity of plasmin was assessed by incubating the analogues with plasmin in the presence of purified fibrin. After incubation for 20 min, lysis in the absence of analogues was $51 \pm 7\%$. This was similar to $48 \pm 9\%$ for EACA (0.6 mM) and was reduced to $23 \pm 9\%$ in the presence of AMCA (0.06 mM).

EACA and AMCA both inhibited the binding of plasminogen to fibrin at concentrations of 0.06 mM and 0.006 mM, respectively, with increasing inhibition at ten-fold higher concentrations (Fig. 2). In contrast, the lysine analogues did not inhibit the initial binding of t-PA to fibrin (Fig. 3).

Studies with the purified components indicated that the major antifibrinolytic effects of the analogues were to inhibit binding of plasminogen to fibrin and conversion of plasminogen to plasmin by t-PA. In addition, AMCA but not EACA at the highest concentration used, appeared to have an inhibitory effect on plasmin-induced proteolysis of fibrin.

Effect of exogenous t-PA on dilute whole blood clot lysis

In the absence of exogenous t-PA the dilute whole blood clot lysis was 325 ± 25 min. The rate of whole blood clot lysis was increased by incorporating exogenous t-PA into the forming clot or by exposing the preformed clot to t-PA (Table 1). In subsequent studies the antifibrinolytic effect of the lysine analogues was determined in dilute whole blood clots that were formed in the presence of exogenous t-PA and in clots that were formed and then exposed to exogenous t-PA. The concentrations of lysine analogues that

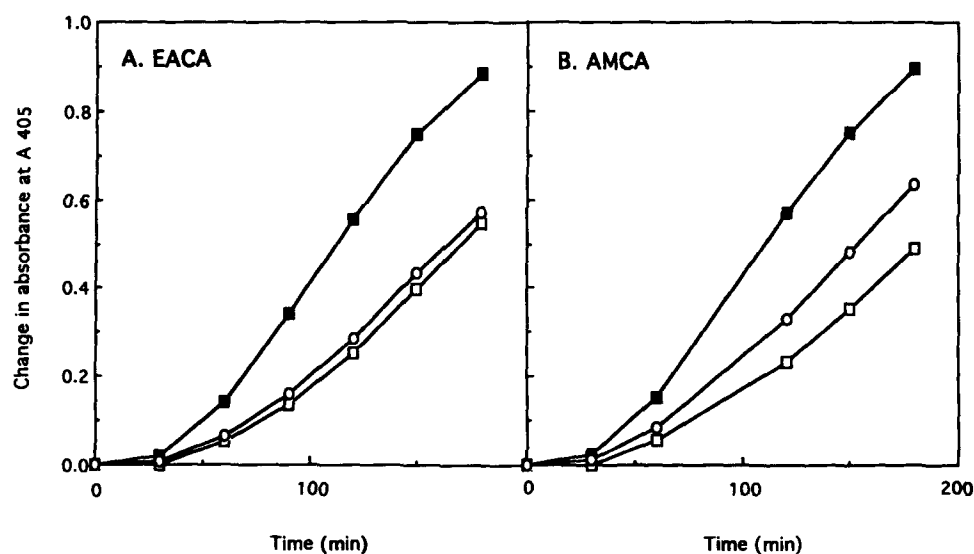


FIG. 1.

Inhibition of t-PA activation of plasminogen by EACA or AMCA. t-PA (2 IU/ml, final) in the presence of buffer, EACA or AMCA was mixed with plasminogen (0.1 mg/ml, final) and the rate of activation to plasmin was monitored with S-2251 (0.25 mM, final) in a 96-well plate (see Methods). Each point is the mean \pm SEM of triplicate experiments. Final concentrations for EACA were 0.06 mM (-○-) and 0.6 mM (-□-); and for AMCA 0.006 mM (-○-) and 0.06 mM (-□-); buffer alone (-■-).

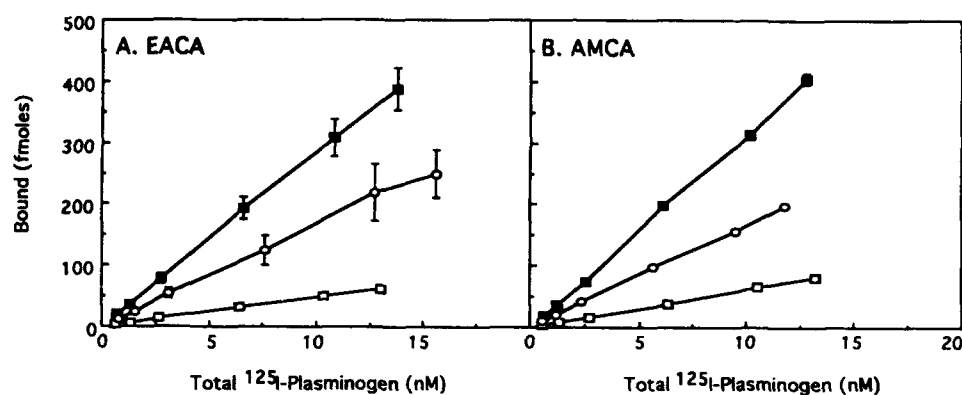


FIG. 2.

Inhibition of plasminogen binding to fibrin by EACA or AMCA. Fibrin matrices were made using 0.2 ml plasminogen-free fibrinogen (1 mg/ml) and thrombin (2 U/ml, final) in 24-well plates. A solution (200 μl) containing ^{125}I -plasminogen with no inhibitor, EACA or AMCA was added to the wells. Bound and free plasminogen were measured as described in Methods. Each point is the mean \pm SEM of 2 to 3 experiments, each performed in duplicate. Final concentrations for EACA were 0.06 mM (-○-) and 0.6 mM (-□-); and for AMCA 0.006 mM (-○-) and 0.06 mM (-□-); buffer alone (-■-).

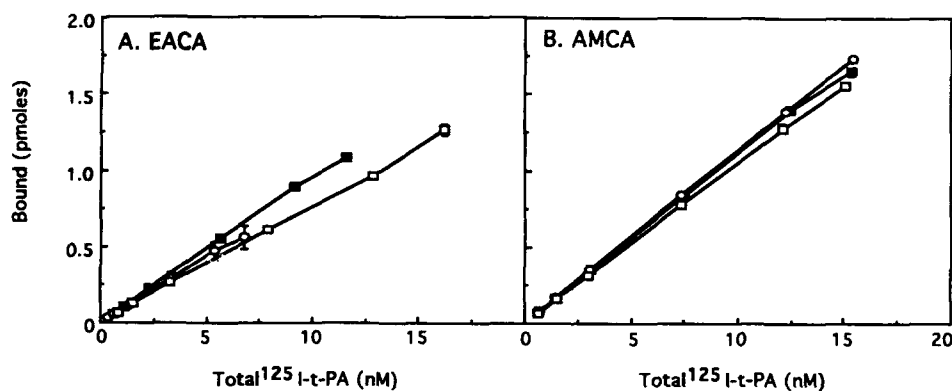


FIG. 3.

Effect of EACA and AMCA on the binding of t-PA to fibrin. A solution (200 μl) containing ^{125}I -t-PA with no inhibitor, EACA or AMCA was added to the wells. Bound and free t-PA were measured as described in Methods. Each point is the mean \pm SEM of 2 to 3 experiments each performed in duplicate. Final concentrations for EACA were 0.06 mM (-○-) and 0.6 mM (-□-); and for AMCA 0.006 mM (-○-) and 0.06 mM (-□-); buffer alone (-■-).

TABLE I.

Lysis Times of Dilute Whole Blood Clots Formed in the Presence of Purified t-PA or Incubated with t-PA after Clot Formation.

t-PA Concentration (IU/ml)	Clot lysis times (min)	
	Forming Clot*	Preformed Clot
3	17 ± 3 [†]	95 ± 1
600	< 2	27 ± 1
1200	< 2	16 ± 1

* t-PA was added before thrombin-induced clot formation.

[†] Values are expressed as the mean ± SEM (n=4) of the clot lysis times.

were used were the lowest and highest levels that would be found in plasma from patients receiving antifibrinolytic treatment (9,11).

Effect of EACA and AMCA on lysis of dilute whole blood clots formed in the presence of exogenous t-PA

In one set of experiments, dilute whole blood containing exogenous t-PA (3 IU/ml, final) was clotted with thrombin and then lysine analogues were added to the supernatant (Fig. 4). EACA at a final concentration of 0.075 mM caused no significant prolongation of lysis time (17 ± 2 min vs 25 ± 5 min, $p=0.20$, Fig. 4A). However, if EACA at this same concentration was included with t-PA before the clot was formed, the lysis time was significantly increased from 17 ± 2 min to 130 ± 17 min ($p<0.01$). A similar result was noted for AMCA at a concentration of 0.0075 mM. These data suggested that t-PA and plasminogen that were incorporated into a forming clot before exposure to lysine analogues were at least partially protected from inhibition.

Antifibrinolytic effect of lysine analogues on dilute whole blood clots that were formed and then incubated with exogenous t-PA

In the following experiments, clots were formed and then incubated with t-PA at a concentration of 3 IU/ml. In one set of experiments, EACA or AMCA was added before clot formation, thereby having a potential inhibitory effect on the binding of plasminogen to fibrin as well as inhibition of t-PA that was added after the clot had formed (Fig. 5). The clot lysis time in the absence of EACA/AMCA was 95 ± 1 min. When either EACA (0.075 mM) or AMCA (0.0075 mM) were added before clot formation, t-PA induced lysis was prolonged by two-fold or greater. A similar degree of inhibition occurred when the EACA or AMCA at these concentrations was added after the clot had formed and before t-PA was added. At higher concentrations, the inhibitory effects of the analogues was more pronounced when added before clot formation.

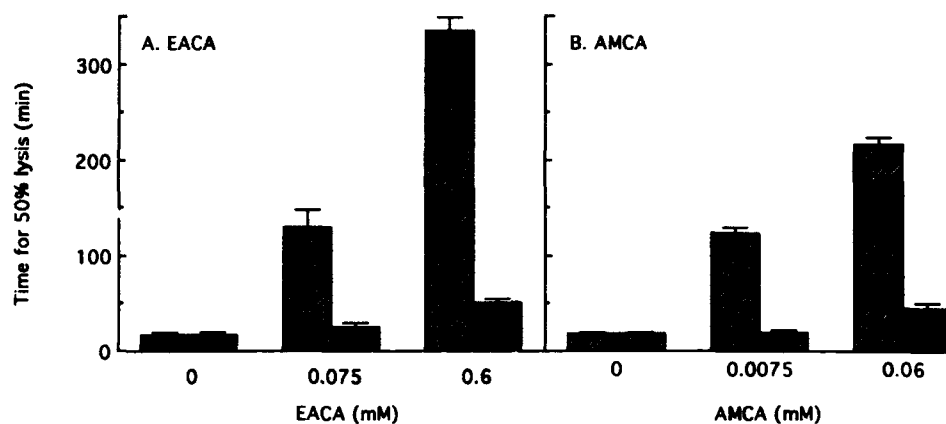


FIG. 4.

Effect of EACA and AMCA on dilute whole blood clot lysis when added before or after clot formation. Freshly drawn whole blood was diluted 1:10 in sodium phosphate buffer, pH 7.4 and mixed with ^{125}I -fibrinogen ($0.02 \mu\text{M}$) and t-PA (3 IU/ml, final). EACA or AMCA was added before or after clot formation. Clot lysis was monitored at 37°C and was expressed as the time at which 50% of the radiolabeled fibrin had been released from the clot (clot lysis time). Each bar is the mean \pm SEM of 6 experiments performed on blood from three donors. The striped bars are the lysis times when EACA or AMCA was added before clot formation and the shaded bars are the lysis times when the lysine analogues were added after clot formation.

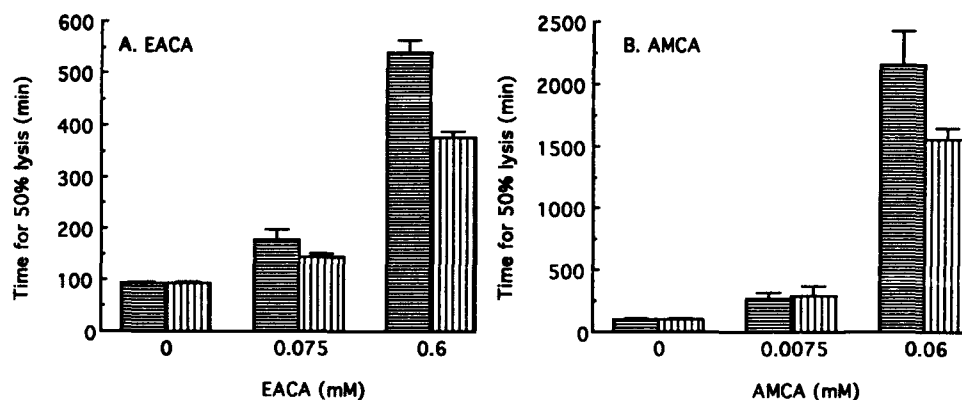


FIG. 5.

Effects of EACA and AMCA on dilute whole blood clot lysis induced by exogenous t-PA added after clot formation. EACA or AMCA was added to dilute whole blood either before (striped) or after (shaded) clot formation. Exogenous t-PA was added to the supernatant of the formed clot after the addition of the lysine analogue. Each bar is the mean \pm SEM of 6 experiments.

The overall effect of lysine analogues on t-PA induced whole blood clot lysis is summarized in Table 2. Lysine analogues were most effective at both low and higher concentrations when included with t-PA before formation of the fibrin clot. Their inhibitory effect was markedly attenuated with clots that were formed in the presence of t-PA and then exposed to EACA or AMCA. A ten-fold lower concentration of AMCA provided an inhibitory effect similar to that of EACA.

DISCUSSION

The present study indicates that low concentrations of lysine analogues exhibit their greatest inhibitory potential if they are present at the time when t-PA is being incorporated into the forming clot. At higher concentrations (0.6 mM for EACA and 0.06 mM for AMCA), they have significant inhibitory effect against preformed clots that are exposed to t-PA. Current recommendations for the use of EACA in patients who have received thrombolytic agents are for the infusion of 0.5 to 1.0 gram/hour, which would provide plasma levels of approximately 0.33 to 0.67 mM (23). These are well above the concentrations needed to inhibit lysis induced by incorporation of t-PA into the forming clot *in vitro*. Other authors have reported the satisfactory use of doses of EACA as low as 6 grams per day in preventing excessive bleeding with patients who have thrombocytopenia (9). The present study suggests that such a dose, which would result in plasma levels of approximately 0.17 mM (23) could still be effective in inhibiting fibrinolysis. The study also suggests efficacy for AMCA at concentrations lower than the recommended levels, which are reported as 0.032-0.064 mM (6). Although the higher concentration of AMCA (0.06 mM) had an inhibitory effect on plasmin-induced proteolysis, EACA at the higher concentration (0.6 mM) showed no inhibition. At these concentrations, no inhibitory effect towards the amidolytic effect of plasmin was shown by these analogues. The ability of lysine analogues to inhibit the proteolytic activity of plasmin without affecting the amidolytic activity has been previously reported (24).

The dilute whole blood clot lysis was chosen as an *in vitro* system that is similar to the clots formed *in vivo* in that both cellular and noncellular components were present in the system. In the dilute whole blood clot lysis assay described in this report, endogenous t-PA comprised less than 5% of the activity of the exogenous t-PA (25). At the concentration of exogenous t-PA (3 IU/ml) used in the present study, the dilute whole blood clots lysed quickly as long as t-PA was incorporated into the forming clot. However, if the clot was formed first and then exposed to t-PA, a 400-fold greater concentration of the enzyme was required to obtain a similar lysis time (Table 1). A partial explanation for this may be the lack of binding sites for t-PA in the whole blood clot that has undergone retraction (26).

Using purified components, investigators have demonstrated that t-PA initially binds to fibrin through the finger domain which is not a lysine binding site (3). Further binding may occur through the kringle 2 domain as fibrin undergoes digestion by plasmin with exposure of C-terminal lysine sites on fibrin (27-29). The kringle 2 domain is also critical for the enhanced activation of plasminogen by t-PA. As fibrin undergoes degradation, the additional lysine binding sites that are available provide for increased binding of plasminogen (30). During the course of fibrinolysis, the lysine analogues would have

TABLE II.

Inhibition of Dilute Whole Blood Clot (DWB) Lysis by EACA and AMCA.

Assay System	Percent Prolongation of Clot Lysis*		
	EACA (0.075 mM)	AMCA (0.0075 mM)	EACA (0.6 mM) AMCA (0.06 mM)
1. DWB (plgn†) and fbgn‡) + t-PA + <u>thrombin</u> clot + EACA/AMCA	50	11	66
2. DWB (plgn and fbgn) + t-PA + EACA/AMCA <u>thrombin</u> clot	665	583	1871
3. DWB (plgn and fbgn) <u>thrombin</u> clot + EACA/AMCA + t-PA	53	201	295
4. DWB (plgn and fbgn) + EACA/AMCA <u>thrombin</u> clot + t-PA	87	180	468
			150
			1106
			1508
			2127

* The percent prolongation was calculated by subtracting the control lysis time (in the absence of lysine analogues) from the value in the presence of the analogues and dividing by the control value.

† - endogenous plasminogen present in DWB

‡ - endogenous fibrinogen present in DWB

continued opportunity to inhibit plasminogen activation by preventing the binding of t-PA and plasminogen to newly exposed lysine sites on fibrin.

The inhibitory activity in a system of purified components cannot necessarily predict what will occur in a more complex system such as the dilute whole blood clot lysis assay. The present study indicates that low levels of analogues are inhibitory in this system and that multiple mechanisms are probably responsible for inhibition at low and at higher concentrations of analogues.

REFERENCES

1. Collen D, Lijnen HR. Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood* 78, 3114-3124, 1991.
2. Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of Fibrin. *J Biol Chem* 257, 2912-2919, 1982.
3. Verheijen JH, Caspers MPH, Chang GTG, de Munk GAW, Pouwels PH, Enger-Valk BE. Involvement of finger domain and kringle 2 domain of tissue-type plasminogen activator in fibrin binding and stimulation of activity by fibrin. *EMBO J* 5, 3525-3530, 1986.
4. Lucas MA, Fretto LJ, McKee PA. The binding of human plasminogen to fibrin and fibrinogen. *J Biol Chem* 258, 4249-56, 1983.
5. Marcus G, DePasquale JL, Wissler FC. Quantitative determination of the binding of epsilon-aminocaproic acid to native plasminogen. *J Biol Chem* 253, 727-732, 1978.
6. Verstraete M. Clinical application of inhibitors of fibrinolysis. *Drugs* 29, 236-261, 1985.
7. Gardner FH, Helmer RE III. Aminocaproic acid. Use in control of hemorrhage in patients with amegakaryocytic thrombocytopenia. *JAMA* 243, 35-37, 1980.
8. Garewal HS, Durie BGM. Anti-fibrinolytic therapy with aminocaproic acid for the control of bleeding in thrombocytopenic patients. *Scand J Haematol* 35, 497-500, 1985.
9. Bartholomew JR, Salgia R, Bell WR. Control of bleeding in patients with immune and nonimmune thrombocytopenia with aminocaproic acid. *Arch Int Med* 149, 1959-61, 1989.
10. Sindet-Pedersen S, Ramstrom G, Bernvil S, Blomback M. Hemostatic effect of tranexamic acid mouthwash in anticoagulant-treated patients undergoing oral surgery. *N Engl J Med* 320, 840-3, 1989.
11. McNicol GP, Fletcher AP, Alkjaersig N, Sherry S. The absorption, distribution, and excretion of epsilon-aminocaproic acid following oral or intravenous administration to man. *J Lab Clin Med* 59, 15-24, 1962.
12. Sane DC, Califf RM, Topol EJ, Stump DC, Mark DB, Greenberg CS. Bleeding during thrombolytic therapy for acute myocardial infarction: mechanisms and management. *Ann Intern Med* 111, 1010-1022, 1989.
13. Racanelli AL, Diemer MJ, Dobies CA, Dubin JR, Reilly TM. Comparison of recombinant plasminogen activator inhibitor-1 and epsilon amino caproic acid in a hemorrhagic model. *Thromb Haemostas* 67, 692-696, 1992.
14. Taylor FB Jr, Muller-Eberhard HJ. Qualitative description of factors involved in the retraction and lysis of dilute whole blood clots and in the aggregation and retraction of platelets. *J Clin Invest* 49, 2068-2085, 1970.
15. Comp PC, Jacocks RM, Taylor FB, Jr. The dilute whole blood clot lysis assay: a

screening method for identifying postoperative patients with a high incidence of deep venous thrombosis. *J Lab Clin Med* 93, 120-127, 1979.

16. Sakata Y, Aoki N. Cross-linking of α_2 -plasmin inhibitor to fibrin by fibrin-stabilizing factor. *J Clin Invest* 65, 290-297, 1980.

17. Castellino FJ, Powell JR. Human plasminogen. *Methods Enzymol* 80, 365-368, 1980.

18. Mosesson MW. The preparation of human fibrinogen free of plasminogen. *Biochim Biophys Acta* 57, 204-213, 1962.

19. Kazal LA, Amsel S, Miller OP, Tocantins LM. The preparation and some properties of fibrinogen precipitated from human plasma by glycine. *Proc Soc Exp Biol Med* 113, 989-994, 1963.

20. Knight LC, Budzynski AZ, Olexa SA. Radiolabeling of fibrinogen using the iodogen technique. *Thromb Haemostas* 46, 593-596, 1981.

21. Fearnley GR, Balmforth G, Fearnley E. Evidence of a diurnal fibrinolytic rhythm; with a simple method of measuring fibrinolysis. *Clin Sci* 16, 645-650, 1957.

22. Wagner OF, de Vries C, Hohmann C, Veerman H, Pannekoek H. Interaction between plasminogen activator inhibitor type 1 (PAI-1) bound to fibrin and either tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). *J Clin Invest* 84, 647-655, 1989.

23. Frederiksen MC, Bowsher DJ, Ruo TI, Henthorn TK, Ts'ao C-H, Green D, Atkinson AJ, Jr. Kinetics of epsilon-amino acid distribution, elimination, and antifibrinolytic effects in normal subjects. *Clin Pharm Therapeut* 35(3), 387-93, 1984.

24. Anonick PK, Vasudevan J, Gonias SL. Antifibrinolytic activities of α -N-acetyl-L-lysine methyl ester, ϵ -aminocaproic acid, and tranexamic acid. Importance of kringle interactions and active site inhibition. *Arterioscler Thromb* 12, 708-716, 1992.

25. Krishnamurti C, Tang DB, Barr CF, Alving BA. Plasminogen activator and plasminogen activator inhibitor activities in a reference population. *Am J Clin Pathol* 89, 747-752, 1988.

26. Kunitada S, FitzGerald GA, Fitzgerald DJ. Inhibition of clot lysis and decreased binding of tissue-type plasminogen activator as a consequence of clot retraction. *Blood* 79, 1420-1427, 1992.

27. van Zonneveld A-J, Veerman H, Pannekoek H. On the interaction of the finger and the kringle-2 domain of tissue-type plasminogen activator with fibrin. Inhibition of kringle-2 binding to fibrin by ϵ -amino caproic acid. *J Biol Chem* 261 14214-18, 1986.

28. Wilhelm OG, Jaskunas SR, Vlahos CJ, Bang NU. Functional properties of the recombinant kringle-2 domain of tissue plasminogen activator produced in *E. coli*. *J Biol Chem* 265, 14606-14611, 1990.

29. Ichinose A, Takio K, Fujikawa K. Localization of the binding site of tissue-type plasminogen activator to fibrin. *J Clin Invest* 78, 163-9, 1986.

30. Fleury V, Angles-Cano E. Characterization of the binding of plasminogen to fibrin surfaces; the role of carboxyl-terminal lysines. *Biochem* 30, 7630-7638, 1991.